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NUCLEOTIDE SEQUENCE AND RESTRICTION SITE ANALYSES IN THREE ISOLATES OF KASHMIR BEE VIRUS FROM *APIS MELLIFERA* L. (HYMENOPTERA: APIDAE)[1,2]

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Abstract.—The 393bp nucleotide sequences of RNA polymerase genes in three isolates of Kashmir bee virus were compared. There was a 97.4% similarity between Canadian and U.S. isolates and an 80.4% or 81.2% similarity between the Australian strain and the two North American isolates. The amino acid sequence similarity between the two North American isolates was 99.2%. The similarities between the Australian isolate and Canadian and U.S. isolates were 97.7% and 96.9%, respectively. These three KBV isolates could also be differentiated by differences in the cleavage sites of the restriction endonuclease *MaeII.*

Key Words: *Apis mellifera,* honey bees, Kashmir bee virus, amino acid sequence, nucleotide sequence, restriction endonuclease, RT-PCR

Kashmir bee virus (KBV) was first isolated from a diseased adult bee of *Apis cerana* Fab. (Bailey and Woods 1977). Strains of KBV have been found in adult bees of *Apis mellifera* L. in Canada, Spain, India, Australia and New Zealand (see Allen and Ball 1995 for further details), Fiji (Anderson 1990) and the U.S. (Bruce et al. 1995, Hung et al. 1995). According to Anderson (1991), KBV is not a virulent pathogen of *A. mellifera.* Bailey and Ball (1991) and Allen and Ball (1995) considered KBV the most virulent virus of all known honey bee viruses. Recently, Hung et al. (1996) reported for the first time that a strain of KBV was found in dead honey bees in the U.S. They also reported the first case of simultaneous infection of acute paralysis virus (APV) and KBV in the same honey bee colony.

The reverse transcription-polymerase chain reaction (RT-PCR) was first used for detecting KBV by Stoltz et al. (1995). However, their paper contains no sequence information of KBV genome other than the two "KBV specific" primers they used in PCR. In this paper, we report the results of our nucleotide sequence and restriction enzyme analyses in three KBV isolates.

MATERIALS AND METHODS

Freeze-dried samples of pupae injected with a South Australian isolate of KBV were supplied by Dr. Brenda V. Ball. A crude KBV extract of 30 pooled bees from Leamington, Ontario, Canada (identified by Dr. Ball) was supplied by Dr. Cynthia Scott-Dupree. One KBV-infected adult worker bee from the same Maryland bee colony used in Hung and Shimanuki (in press a)

[1] The nucleotide sequence data reported here have been registered with GenBank under Accession Nos. AF027125, AF034541, and AF034542.

[2] Mention of any proprietary product in this publication does not constitute an endorsement for use of this product by the USDA.

was also included in this study. Freeze-dried pupae were rehydrated and homogenized in 0.45 mL of 0.01 M potassium phosphate buffer (pH 6.7). The same amount of buffer was used to homogenize the adult worker bee. After centrifugation at ca. 4,000 g for 15 minutes, direct RT-PCR was carried out with 2 μL of supernatant from either the homogenized sample or the crude extract as described by Hung and Shimanuki (in press b). The primer pair 5′-GATGAACGTCGACCTATTGA-3′ and 5′TGTGGGTTGGCTATGAGTCA-3′ (Stoltz et al. 1995) was used in PCR with each primer at a final concentration of 0.2 μM.

The RT-PCR reaction product was purified with the High Pure PCR Product Purification Kit (Boehringer Mannheim). The dye-terminator sequencing of the purified PCR product was carried out on an ABI DNA sequencer (model 373a) at the DNA Sequencing Facility of the University of Maryland Center for Agricultural Biotechnology. Both DNA strands were sequenced three times to confirm the accuracy of the sequence data. Sequence data were edited and analyzed using DNASIS™ for Windows® Version 2.5 (Hitachi Software, South San Francisco, CA). DNASIS™ was also used in the multiple alignment and the restriction site search.

Restriction endonuclease (RE) digestion was carried out with 3μL of purified PCR products, 5 μL of 2× RE buffer, 1μL of sterile water and 1.5 μL (= 3 units) of the RE, *MaeII* (Boehringer Mannheim), at 50°C for 24h. The digested DNA was analyzed by electrophoresis through a 1.5% (w/v) agarose gel and stained in ethidium bromide (0.5 μg/mL). After destaining, DNA bands were visualized with a UV transilluminator.

Results

The RT-PCR of both the Australian and Canadian KBV isolates generated a 393 bp product which is the same size as the U.S. KBV reported by Hung and Shimanuki (in press a). Fig. 1 shows the edited sequences of the 393 bp PCR product of both isolates and the sequences of these two segments aligned with that of the U.S. KBV. Multiple alignment of these sequences showed a 97.4% similarity between Canadian and U.S. isolates. The similarities between the Australian isolate and Canadian and U.S. isolates were 80.4% and 81.1%, respectively.

All three sequences contain a single large open reading frame (deduced amino acid sequence not shown). Although there are 43 miss-matched triplet groups, only four of them with miss-match resulted in amino acid coding differences (Fig. 1, triplet groups in bold face and shaded). These are at positions 123 (ACT for threonine; GCA for alanine), 303 (GCT for alanine; CCA for proline), 312 (TGC for cysteine; GCT for alanine), and 336 (ATG for methionine; GTG for valine). Therefore, the amino acid sequence similarity between the two North American isolates was 99.2%. The similarities between the Australian isolate and Canadian and U.S. isolates were 97.7% and 96.9%, respectively.

The DNASIS™ database was used in the search for restriction enzymes with one cutting site at these 393bp amplicons that will result in a restriction fragment length polymorphism (RFLP). We found that these three isolates could also be differentiated by the differences in *MaeII* restriction endonuclease cleavage sites. No cleavage site was found in the Australian isolate. As shown in Fig. 1, the U.S. and Canadian isolates each possessed a different *MaeII* restriction site: in the U.S. isolate at triple #83 and in the Canadian at triplet #107. Fig. 2 shows the RFLP of *MaeII*. The "extra" low molecular weight fragment (about 100bp) in the U.S. sample was a nonspecific PCR product from the homogenate of the adult worker bee.

Discussion

The size of the 417 bp product reported for Canadian KBV by Stoltz et al. (1995)

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                          11          20          29          38          47
KBVAU01 --GA CCT ATT GAA AAG GTT AAT CAA TTG AAA ACA CGA GTA TTC TCA AAT
KBVCA01 --.. ... ... ... ..A ... ... ... ... ... ... ... ... ... ... ...
KBVUS01 TC.. ... ... ... ..A ... ... ... ... ... ... ... ... ... ... ...
                     56          65          74          83          92
KBVAU01   GGA CCA ATG GAT TTC TCT ATA GCT TTT CGA ATG TAT TAT TTG GGC TTT
KBVCA01   ... ... ... ..C ... ..A ..T ..G ... ... ... ... ... ... ... ...
KBVUS01   ... ... ... ..C ... ..A ..T ..G ... ... ... ... ... ... ... ...
               101         110         119         128         137
KBVAU01   ATA GCT CAT TTA ATG GAA AAT CGA ATT ACT AAT GAG GTA TCT ATT GGA
KBVCA01   ... ... ...   G ... ..G ... ... ... G.A ... ..A ..G ... ... ...
KBVUS01   ... ... ... ..G ... ..G ... ... ... G.A ... ..A ..G ... ... ...
          146         155         164         173         182         191
KBVAU01   ACG AAT GTG TAT TCT CAG GAC TGG AGT AAA ACT GTT CGC AAA TTG ACC
KBVCA01   ... ... ..T ..C ..A ..A ... ... ... ... ... ... A.G ... ..A ...
KBVUS01   ... ... ..T ..T ..A ..A ... ... ... ... ... ... A.G ... ..A ...
                    200         209         218         227         236
KBVAU01   AAA TTT GGA AAT AAA GTT ATT GCG GGT GAT TTT TCA ACT TTT GAT GGA
KBVCA01   ... ... ... ... ... ..A ... ..T ... ... ... ... ... ... ... ...
KBVUS01   ... ... ... ... ... ..A ... ..T ... ... ... ... ... ... ... ...
               245         254         263         272         281
KBVAU01   TCA CTG AAT GTA TGT ATT ATG GAA AAA TTT GCG GAT TTA GCG AAT GAG
KBVCA01   ... ..A ..T ..T ..C ... ... ... ... ... ..A ... ... ..T ... ...
KBVUS01   ... ..A .AC GTT ..C ... ... ... ... ... ..A ... ... ..T ... ...
          290         299         308         317         326         335
KBVAU01   TTT TAC GAT GAT GGT GCT GAG AAT TGC CTG ATT AGG CAT GTG TTA TTG
KBVCA01   ... ... ... ..C ..A C.A ..A ... GCT T.. ..A CGT ... ..A C.. ...
KBVUS01   ... ... ... ..T ..A C.A ..A ... GCT T.. ..C C.T ... ..A T.. ...
                    344         353         362         371         380
KBVAU01   ATG GAT GTG TAT AAT TCT GTA CAC ATT TGC AAT GAC TCT GTC TAT ATG
KBVCA01   A.. ... ... ..C ... ... ..A ..T ... ..T ... ..T ..A ..A ..C ...
KBVUS01   G.. ... ... ..C ... ... ..G ..T ... ..T ... ..C ..A ..A ..C ...
               389
KBVAU01   ATG ACT CAT A
KBVCA01   ........... .
KBVUS01   ..........- -
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Fig. 1. Multiple nucleotide sequence alignment of the 393bp RT-PCR product of Australian (KBVAU01, GenBank AF034541), Canadian (KBVCA01, GenBank AF034542) and U.S. (KBVUS01, GenBank AF027125) KBV isolates. Dots represent bases the same as in KBVAU01. The recognition sequence of the restriction enzyme *MaeII* (A↓CGT) is underlined and shaded. Triplets that coded for different amino acids between the samples are in bold face and shaded.

was based on their estimation using the molecular weight DNA markers. Although we also used their two primers in PCR, the 393 bp length of the sequenced amplicon reported here represents the actual count of bases. Stoltz et al. (1995) did not report the nucleotide sequence of the estimated 417 bp amplicon of their Canadian KBV sample. Our search in the available databases has failed to find any honey bee virus sequence data deposited by them. Therefore, no comparison can be made between our Canadian sample and theirs.

We have shown that the "traditional" RT-PCR protocols used in Hung and Shimanuki (in press a) and the direct RT-PCR protocol of Hung and Shimanuki (in press b) used in this report produced similar results, both in terms of the size of amplicon and the nucleotide sequence (Fig. 1). The latter protocol can circumvent the time-consuming virus and RNA purification steps.

According to Bruce et al. (1995, Table 1) there are three "serological" strains of KBV in each of the seven states they surveyed. Fig. 1 from their paper, however,

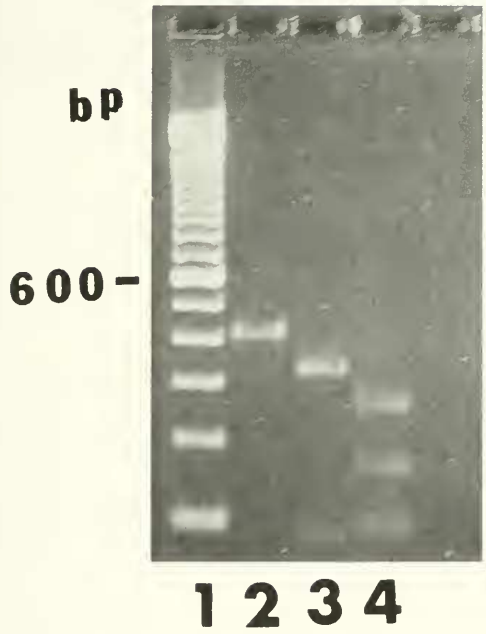

Fig. 2. Restriction fragment length polymorphism of the 393bp PCR products in three isolates of KBV. DNA products were digested with the restriction enzyme *MaeII*. Lane 1, 100bp DNA ladder; lane 2, Australian KBV; lane 3, Canadian KBV; lane 4, U.S. KBV.

does not demonstrate that "serologically distinct strains of the virus exist in the U.S." Allen and Ball (1995) divided strains of KBV into two serological groups: the Canadian and Spanish strains formed one group and the Indian, South Australian and New Zealand strains formed another group. As Allen and Ball (1995) further pointed out, "studies on the genomes of both APV and KBV should help to clarify these relationships." Sequence data presented here will serve as reference in further nucleotide sequence analyses of multiple APV and KBV isolates from different localities throughout North America.

Restriction enzyme analysis of PCR-amplified fragments has been used in differentiating isolates of cucumber mosaic virus isolates (Rizos et al. 1992) and ovine lentiviruses (Leroux et al. 1995). As shown in Fig. 2, these three isolates of KBV can be differentiated from each other by the RFLP of *MaeII*. RT-PCR and RFLP analysis of PCR-amplified fragments, therefore, should provide a quick and easy tool to distinguish these three KBV isolates without carrying out sequence analysis.

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